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13. ABSTRACT *(Maximum 200 Words)*

Recent studies have correlated higher levels of 5-hydroxy- methyl-2'-deoxyuridine (HMdU), an oxidized DNA base, with an increased risk of cancer when compared to controls. HMdU has been proposed to be a biomarker of breast cancer risk. We have developed a simple and sensitive fluorometric HPLC method for HMdU quantitation, which will be useful in the epidemiological studies. This method utilizes 7-dimethylamino-coumarin-3-acetic-acid (DMACA) as the fluorescent probe for the pre-column derivatization in anhydrous acetonitrile in the presence of catalyst at 65°C for 1 h. The method was optimized for various parameters involved in reaction. The linearity of the fluorescent labeling method was established by labeling low levels of HMdU (in the range of 5 pmol to 0.2 pmol). Commercially available salmon testes DNA was utilize to develop the method for HMdU quantitation from DNA, which is in final stage of development. To quantitate HMdU, DNA was enzymatically digested to nucleosides, which are well separated by HPLC-1. After passing through cation exchanger, HMdU fractions are concentrated, subjected to coumarin post-labeling and quantitated using normal phase HPLC-2 with fluorescence detection . This method will utilize to qunatitate HMdU from blood clots.

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1. Introduction:

Increasing evidence implicates oxidative DNA damage as playing an important role in development of breast as well as other types of cancer. Our hypothesis is that oxidative DNA damage begins much earlier than the clinical manifestation of breast cancer. Recently more and more studies correlate the elevated 5-hydroxymethyl-2'-deoxyuridine (HMdU - an oxidized DNA base) levels with an increased risk of cancer when compared to controls (Djuric *et al.,* 1996 & 2001, Simon *et al.*, 2000) HMdU has been proposed to be a biomarker for breast cancer risk. Our laboratory has shown that sera of healthy women with a family history of breast cancer and women apparently diagnosed with breast cancer several years after blood donation had elevated amounts of anti-HMdU antibodies (Frenkel *et al.,* 1998). This suggests that in the early stages of cancer development cells are under oxidative stress, which is manifested by significant HMdU formation. To test our hypothesis, we proposed to develop a new fluorescence post-labeling method to quantitate HMdU levels from biological samples. Although methods are available for detection and quantitation of HMdU from various biological samples, either it is expensive (GC/MS - Gas Chromatography/ Mass Spectrometry) or have problems with radioactive waste disposal. The method will be developed for DNA from WBC and will be utilized to detect and quantitate HMdU from blood clots obtained in conjunction with the New York University Women's Health Study.

In a previous report, we described the fluorescent labeling of HMdU, using 7-dimethyl-amino-coumarin-3-acetic acid (DMACA) in the presence of 4-pyrolidinopyridine (PPy) and 4,4'-dicyclohexyl-carbodiimide (DCC), which showed the labeling being done mainly on primary -OH group of nucleoside. Normal phase HPLC (silica) was used to analyze the fluorescent labeled product. The detection limit for the purified fluorescent dU and HMdU products was found to be 0.01 fmol. Our fluorescence labeling method was optimized for HMdU at a 1 nmol level by varying parameters involved in the reaction.

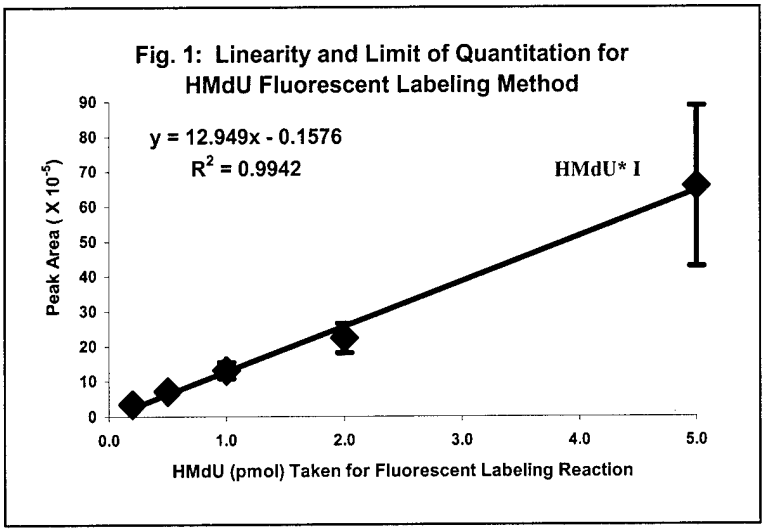
2. Studies and Results

The limits of detection and quantitation for the HMdU fluorescent labeling method was obtained by using progressively smaller amounts of HMdU from 100 pmol to 0.1 pmol. 5 pmol 2'-deoxyuridine (dU) as a reporter molecule was added to the labeling reaction containing 5 pmol or less HMdU to calculate and compensate losses during the fluorescent labeling reaction. In a typical fluorescent labeling reaction, pre-aliquoted HMdU is reconstituted with anhydrous acetonitrile and amounts ranging from 5 pmol to 0.1 pmol were transferred to polypropylene eppendorff tubes. 5 pmol dU (reporter molecule) was added along with 100 nmol DMACA (fluorescence probe) and 5 nmol 4-pyrrolidino pyridine prepared in anhydrous acetonitrile. Finally 1 μmol DCC was added and the reaction mixture was incubated at 65°C for 1 hr.

After incubation, the reaction mixture was dried and pre-purified using silica SPE cartridge before analysis on normal phase HPLC. We start analyzing 50% of the labeling reaction for less than 5 pmol of HMdU labeling reaction, since we wanted to simulate the same condition as the quantitation of HMdU from DNA. The baseline signal becomes noisier and we loose the sensitivity of detection as well as quantitation for lower levels of HMdU. We found that at lower levels of HMdU, various factors (viz. sample preparation, reaction pre-purification and drying after pre-purification before HPLC analysis, condition of HPLC column aging and tailing effect) affect the detection and quantitation of the HMdU.

As discussed in our previous report, HMdU fluorescent labeling gives mainly two fluorescent peaks assigned as HMdU* I and HMdU* II, since HMdU has two primary –OH groups.

When low amounts of HMdU (1pmol or less) were taken for fluorescence labeling, we observed that the HMdU* I gave a linear response as compared to HMdU* II. Since HMdU* II peak elutes later in HPLC analysis, in an attempt to resolve earlier dU* and HMdU* I peaks, it suffers more from peak tailing and also noise from the background fluorescence which does not allow proper quantitation. Hence, we used the HMdU* I peak for linearity of quantitation for HMdU.



The limit of quantitation for the HMdU fluorescence labeling method was obtained in the range of 5 pmol to 0.2 pmol as shown in Fig 1. 5 pmol dU is added as a reporter molecule during labeling reaction. The peak area for HMdU* I is calculated in relation to dU* peak. The losses during fluorescent labeling is compensated by comparing dU* peak area from the labeling reaction to the pure 5 pmol dU*. The peak area for pure 5 pmol dU* is considered as 100% and dU factor is calculated as:

$$\text{dU factor} = \frac{\text{dU* peak area from pure 5pmol dU*}}{\text{dU* peak area from labeling reaction of 5 pmol dU}}$$

The losses for HMdU during labeling reaction compensated by multiplying the value for HMdU* I peak area from HPLC with dU factor. The linearity curve displays the average value of HMdU* I of different individual HMdU labeling reactions at each level. The standard deviation is displayed as error bars.

Application of Fluorescent post-labeling for HMdU from DNA

In order to apply this method to quantitate HMdU from DNA samples, commercially available salmon testes DNA (Sigma, MO) was used. The use of naked DNA to develop a method for HMdU quantitation was not proposed in the initial proposal. However, employing commercially available DNA for our study will allow us to develop a method applicable to DNA isolated from various sources while saving the time required for such isolation.

Salmon testes DNA was dissolved in water at a concentration of 5 μg/μl and aliquoted into 100 μg sample. Each vial was flushed with nitrogen and stored at –80°C.

Pre-aliquoted salmon testes DNA was enzymatically hydrolyzed according to an improved DNA digestion method developed in our lab (Huang et al. in press). In a typical hydrolysis, 100μg DNA is incubated 100 mM NaCl, 10 mM Mg^{+2}, 10mM Tris (pH 7.4), 40 μM DTPA, and 40 units of DNase I in a final volume of 100μl at 37°C for 30 min. After adjusting the pH to 5.2 with 1μl of 3M sodium acetate (pH 5.2), the fragmented DNA is digested with 1μl Nuclease P1 (NP1 1unit/μl) for 1hr. The pH was brought back to neutral with 10μl of 1M Tris.HCl (pH 8.0) and alkaline phosphatase was added followed by 30 min incubation at 37°C. Finally, phosphodiesterase I & II (0.001 unit/ μl each) was added for to the reaction mixture and incubated for more 30 min at 37°C

to ensure complete hydrolysis of DNA to nucleosides. After 2.5h digestion, the reaction mixture was centrifuged through Ultrafree-MC membrane (NMWL 5,000 Millipore, MA) to filter out enzymes before HPLC-1.

2'dexoyribonucleosides were separated using reverse phase HPLC (C_{18}) as described in our previous report. The condition for separation was altered due to an as yet unidentified impurity from DNA which was eluting very closely to the HMdU fraction. This unidentified peak becomes visible when digested DNA is kept at room temperature for extended periods. We attempted to examine whether the unidentified product was either a degradation or deamination product of one of the four major 2'deoxyribonucleosides of DNA by subjecting each of the nucleosides (dA, dC, dG and dT) to similar enzymatic hydrolysis. None of nucleosides, when hydrolyzed, resulted in formation of a product corresponding to the unidentified impurity. We changed the HPLC gradient elution by slowing the gradient and organic phase in HPLC separation (from acetonitrile to methanol) to acquire a better separation of the HMdU fraction from other peaks.

Pre-purification of HMdU fluorescent labeling method also needed to be modified. When the HMdU fractions from salmon testes DNA was subjected to fluorescent post-labeling, a significantly fluorescent peak seemed to appear before and overwhelm the florescent dU (dU*) peak. The conditions for solvent B as described in our previous report was changed from 3 ml 5% methanol/ 95% methylene chloride to 6 ml 4 % methanol/ 96% methylene chloride.

Furthermore, HPLC analysis of the fluorescent post-labeling of the HMdU from salmon testes DNA displayed another fluorescent peak between the dU* and fluorescent labeled HMdU peak (HMdU* I). Since this peak is between dU* and HMdU* I, it could not be resolved by altering the pre-purification method. Therefore, we tried to purify the HMdU fraction obtained from HPLC – 1 by using a different solid phase extraction (SPE) cartridge. After several trials we succeeded in eliminating up to 95% of the fluorescent peak which appears before HMdU* I by passing the HMdU fraction, from HPLC of enzymatically digested DNA, through SCX SPE (action exchange) cartridge (Alltech Associates, IL). Since the SCX SPE cartridge retains one of the impurities in the HMdU fractions, we have incorporated this SCX purification of the HMdU fraction before the fluorescent post-labeling reaction.

We are in the final stage of method development for HMdU quantitation from salmon testes DNA. The HMdU levels quantitated by fluorescent labeling method will be compared with the HMdU levels quantitated by GC/MS method

DNA from Blood:

Before applying this method to quantitate HMdU from precious and irreplaceable blood clots obtained in conjunction with the New York University Women's Health Study, we intend to optimize the our fluorescence post-labeling method to quantitate HMdU from DNA of WBC. The use of DNA from whole blood for HMdU quantitation was not in the proposal, but its use will allow us to 1) optimize the HMdU quantitation method DNA from white blood cells (WBC) without sacrificing invaluable blood clots and 2) will allow us to correlate levels of HMdU between whole blood and blood clots from the same individual.

The blood was drawn from healthy volunteers of our research lab, after formal consent from individual members was obtained under the approved Human Subject Protocol (protocol # H-6979-05) by IBRA. WBCs were isolated from blood according to the method (Frenkel et al.1986), which isolates all WBC (including monocytes and neutrophils) from blood.

The DNA was isolated from WBC according to an improved protocol for DNA isolation from biological samples developed in our lab (Huang et al. in press). WBC from whole blood were washed twice in 3 ml ice-cold nuclei isolation solution [10 mM Tris-HCI, pH 8.0, 1% (v/v) Triton X-100, 0.32 M sucrose, 0.2 mM EDTA, 0.1 mM DTPA, and 5 mM $MgCl_2$] to remove cytoplasm and red blood cells (RBC). Addition of DTPA was intended to chelate iron released from RBC or cytoplasm, thus avoiding possible spurious DNA oxidation. The nuclei were suspended in 1.5 ml RNase solution (10 mM Tris-HCI, pH 8.0, 5 mM EDTA, and 0.1 mM DTPA). One µl DNase-free RNase was then added and the mixtures were incubated at 37^0C for 0. 5 h. The nuclei suspension was then mixed with 1. 5 ml cell lysis solution [10 mM Tris-HCI, pH 8.0, 1% (v/v) SDS, 5 mM EDTA and 0.1 mM DTPA]. Proteinase K (20 µl of 20 µg/µ1) was added and incubated at 55^0C for 1 h, followed by an additional 10 µl Proteinase K and incubation for 2 more hours. After cooling the solution on ice, one ml protein precipitation solution (Puregene®, Gentra Systems, MN) was added and shaken vigorously. Precipitated protein was removed by centrifugation and the supernatants were extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). The upper aqueous phase was treated again with 1 µl DNase-free RNase for 15 min at 37^0C. Elimination of RNA is very important in enhancing the efficiency of enzymatic digestion and in obtaining clean HPLC profiles detected by UV. The aqueous phase was extracted once more with an equal volume of chloroform:isoamyl alcohol. DNA was precipitated in the aqueous phase with equal volume of ice-cold isopropanol and stored at -20^0C overnight. After centrifugation, DNA was washed twice with 70% ethanol and dissolved in HPLC grade water. Purity and amounts of DNA were obtained by measuring the ratio of 260/280. The DNA was divided into 100 µg aliquots, flushed with nitrogen and stored at -80^0C.

From 20 ml blood sample we were able to isolate approximately 350µg DNA. Since WBC varies from individual to individual, the yield of DNA also varies.

The method for DNA isolation from blood clots is being developed in our lab so as to minimize background oxidation to normal nucleosides during DNA isolation. This method is in its final stage of development and will be utilized to isolate DNA from blood clots.

Significance:

The most significant finding stemming from the studies presented in this progress report is that this method was developed to label HMdU up to 0.2 pmol. The linearity of quantitation of the HMdU fluorescence labeling method is in the range of 5 to 0.2 pmol.

PLAN:

We intend to further optimize this method to quantitate HMdU using DNA from WBC's. Further, we intend to correlate the HMdU levels isolated from whole blood and blood clots. Finally, HMdU will be quantitated from blood clots obtained from NYU WHS.

Key Research Accomplishments to Date:

- Fluorescence labeling of HMdU was accomplished by acylation with 7-dimethylamino coumarin-4-acetic acid (DMACA).
- Limit of quantitation of HMdU fluorescent labeling method is up to 0.2 pmol. The quantitation is linear in the range from 5 to 0.2 pmol for HMdU labeling reaction.

Reportable Out come: N/A

Conclusion:

We have developed a sensitive fluorescence post-labeling method for quantitation of HMdU, an oxidized DNA base, to detect and quantitate up to 0.2 pmol HMdU. The HMdU quantitation is linear in the range from 5 to 0.2 pmol. This method will be utilized for analysis of DNA isolated human WBC, as a measure of oxidative DNA damage. HMdU quantitation from DNA isolated from biological samples is still at developmental stage. Samples from different sources exhibit different background impurities, which lead to unidentified fluorescent peaks during HMdU quantitation. These impurities may be inherent or artifactual, arising during the isolation process.

References:

Djuric Z., Heilbrun L. K., Lababidi S., Berzinkas E., Simon M. S., Kosir M. A. Levels of 5-hydroxy-methyl-2'-deoxyuridine in DNA from blood of women scheduled for breast biopsy. *Cancer Epidemiol Biomarkers Prev,* **10**,147- 49 (2001).

Djuric Z., Heilbrun L. K., Simon M. S., Smith D., Luongo D. A., LoRusso P., Martino S. Levels of 5-Hydroxymethyl-2`-Deoxyuridine in DNA from Blood as a marker of breast cancer. *Cancer,* **77**, 691-696 (1996).

Frenkel K., Karkoszka J., Glassman T., Dubin N., Toniolo P., Taioli E., Mooney L. A., Kato I. Serum autoantibodies recognizing 5-hydroxymethyl-2'-deoxyuridine, an oxidized DNA base, as biomarkers of cancer risk in women. *Cancer Epidemiol Biomarkers Prev,* **7,** 49-57 (1998)

Frenkel K., Chrzan K., Troll W., Teebor G. W., Steinberg J. J. Radiation-like modification of bases in DNA exposed to tumor promoter- activated polymorphonuclear leukocytes. *Cancer Res,* **46**, 5533-40 (1986).

Huang X., Powell J., Mooney L., Li C. L., Frenkel K. Importance of complete DNA digestion in minimizing variability in the analysis of 8-oxo-dG as a biomarker of cancer risk, *Free Radic Biol Med,* (in press)

Simon M. S., Heilbrun L.K., Stephens D., Lababidi S., Djuric Z. Recruitment for a pilot case control study of oxidative DNA damage and breast cancer risk. *Am J Clin Oncol* , **23**, 283-287, (2000)